Received: 5 June 2023 | Revised: 10 July 2023 | Accepted: 9 October 2023

DOI: 10.1002/pld3.542

RESEARCH ARTICLE

CBSX2 is required for the efficient oxidation of chloroplast redox-regulated enzymes in darkness

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Funding information
This work was supported by the Shanghai Engineering Research Center of Plant Germplasm Resources (17DZ2252700) and Taishan University doctoral research start-up fund project (5013230130).

Abstract

Thiol/disulfide-based redox regulation in plant chloroplasts is essential for controlling the activity of target proteins in response to light signals. One of the examples of such a role in chloroplasts is the activity of the chloroplast ATP synthase (CF_oCF_1), which is regulated by the redox state of the $CF_1\gamma$ subunit and involves two cysteines in its central domain. To investigate the mechanism underlying the oxidation of $CF_1\gamma$ and other chloroplast redox-regulated enzymes in the dark, we characterized the Arabidopsis *cbsx2* mutant, which was isolated based on its altered NPQ (non-photochemical quenching) induction upon illumination. Whereas in dark-adapted WT plants $CF_1\gamma$ was completely oxidized, a small amount of $CF_1\gamma$ remained in the reduced state in *cbsx2* under the same conditions. In this mutant, reduction of $CF_1\gamma$ was not affected in the light, but its oxidation was less efficient during a transition from light to darkness. The redox states of the Calvin cycle enzymes FBPase and SBPase in *cbsx2* were similar to those of $CF_1\gamma$ during light/dark transitions. Affinity purification and subsequent analysis by mass spectrometry showed that the components of the ferredoxin-thioredoxin reductase/thioredoxin (FTR-Trx) and NADPH-dependent thioredoxin reductase (NTRC) systems as well as several 2-Cys peroxiredoxins (Prxs) can be co-purified with CBSX2. In addition to the thioredoxins, yeast two-hybrid analysis showed that CBSX2 also interacts with NTRC. Taken together, our results suggest that CBSX2 participates in the oxidation of the chloroplast redox-regulated enzymes in darkness, probably through regulation of the activity of chloroplast redox systems in vivo.

KEYWORDS
CBSX2, $CF_1\gamma$ subunit, NTRC, peroxiredoxin, photosynthesis, thioredoxin

1 | INTRODUCTION

To ensure the efficiency of photosynthesis and other metabolic reactions, the activity of chloroplast enzymes needs to be flexibly controlled in response to changes in the light environment. Chloroplast redox regulation based on dithiol-disulfide exchange is an important regulatory mechanism for rapid adaptation of chloroplasts to photometabolism (Cejudo et al., 2021). During photosynthesis,

reducing power generated by the electron transport chain in the thylakoids can be transferred from ferredoxin (Fd) to Trx via Fd-thioredoxins (Trx) reductase (FTR). Then, Trx transfers the reducing power to target enzymes such as the four Calvin–Benson cycle enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), fructose 1,6-bisphosphatase (FBPase), sedoheptulose 1,7-bisphosphatase (SBPase) and phosphoribulokinase (PRK) as well as NADP-malate dehydrogenase (MDH), glucose 6-phosphate dehydrogenase (G6PDH) and chloroplast ATP synthase (Gütle et al., 2017). In darkness, these target enzymes are rapidly inactivated by oxidation through formation of disulfide bonds.

Chloroplast ATP synthase on the thylakoid membrane belongs to the F-type ATP synthases containing the perimembranous part CF_1 and the intrinsic part CF_o. During photosynthesis, accumulation of the protons in the thylakoid lumen leads to the generation of a proton motive force (*pmf*) across thylakoid membranes, which powers CF_oCF_1 to synthesize ATP in the chloroplast stroma (Hahn et al., 2018; Hisabori et al., 2013). Like other F-type ATP synthases, chloroplast ATP synthase operates fully reversibly. Indeed, while chloroplast ATP synthase normally generates ATP during photosynthesis, it can also catalyze the hydrolysis of ATP when the *pmf* is insufficient to drive ATP synthesis (Hisabori et al., 2002). To prevent the waste of ATP in chloroplasts, regulation of chloroplast ATP synthase activity plays an important role during the normal operation of photosynthesis and in darkness (Davis & Kramer, 2020; Hahn et al., 2018).

The activity of chloroplast ATP synthase is mainly regulated by changes of the redox state of the $CF_1\gamma$ subunit, which is thought to act as a "switch" that enables the activity of chloroplast ATP synthase to be rapidly switched off upon a transition from light to dark (Hahn et al., 2018; Nalin & McCarty, 1984). Redox regulation of the γ subunit of chloroplast ATP synthase is mediated by a stretch of 9-amino acids containing two cysteine (Cys) residues (Cys199-Cys205 in Arabidopsis) (Nalin & McCarty, 1984). Under light conditions, Trxs receive electrons from the photosynthetic electron transport chain through the FTR and then reduce the disulfide bond formed between the two Cys residues in the γ subunit, resulting in a reduced state of these Cys residues and in the activation of ATP synthase. Under dark conditions, the disulfide bond of the γ subunit is formed by oxidation to prevent the ATP synthase from hydrolyzing ATP (Konno et al., 2000; Yang et al., 2020).

It is well established that upon illumination chloroplast Trxs mediate the transfer of reducing equivalents from the photosynthetic electron transfer chain through Fd and FTR to the target enzymes. Chloroplast Trxs include five subtypes of isoforms in Arabidopsis: *m* (four isoforms), *f* (two isoforms), *y* (two isoforms), *x*, and *z* (Cejudo et al., 2021; Nikkanen & Rintamäki, 2019; Thormählen et al., 2017). It has been shown that $CF_1\gamma$ can be reduced by both *f*-type and *m*-type Trxs (Schwarz et al., 1997; Sekiguchi et al., 2020). Chloroplasts also harbor an NADPH-dependent redox pathway. NTRC (NADPH-Trx reductase C) is unique to oxygenic photosynthetic organisms and consists of the NTR domain with a Trx at its C-terminus (Serrato et al., 2004). As NTRC uses NADPH as a source of reducing power, it works independently of light, unlike the Trx pathway (Cejudo et al., 2021; Pérez-Ruiz et al., 2006, 2017). It may contribute to reduce $CF_1\gamma$ under low light conditions (Carrillo et al., 2016).

It is known that $CF_1\gamma$ is oxidized under dark conditions. Recently, two types of Trx-like proteins, Trx-like 2 (TrxL2) and atypical Cys His-rich Trx (ACHT), were shown to be oxidation factors of Trx-targeted proteins, such as fructose-1,6-bisphosphatase, Rubisco activase, and the $CF_1\gamma$ subunit of ATP synthase (Yokochi et al., 2021). Peroxiredoxins (Prx) are thiol peroxidases that are highly conserved throughout all biological kingdoms. Chloroplasts of plants contain three types of Prx, the classic 2-cysteine peroxiredoxin (2-Cys Prx), a bacteroferritin-comigratory protein homolog Prx-Q, and a type II peroxiredoxin named Prx-II (Dietz, 2003). It has been shown that the 2-Cys Prx A and B participate in the oxidation of thiol groups in the dark (Ojeda et al., 2018; Vaseghi et al., 2018; Yoshida et al., 2018). In the absence of photosynthetic electron transport, Trx may mediate the oxidation of reduced $CF_1\gamma$ and transfer reducing equivalents to hydrogen peroxide through the activity of 2-Cys Prx (Cejudo et al., 2019).

Cystathionine β-synthase (CBS) domain-containing proteins are widely distributed in bacteria, yeasts, animals, and plants (Ignoul & Eggermont, 2005; Kushwaha et al., 2009). The CBS domain consists of approximately 60 amino acids forming two α-helices and three β-strands. In the CBS-containing proteins, the CBS domains are generally found as tandem repeats, particularly in pairs or quads (Bateman, 1997). Tandem paired CBS modules can bind adenosine derivatives, such as ATP, AMP, and SAM (S-adenosyl-L-methionine), and regulate the function of related enzymes or other domains by binding these ligands (Baykov et al., 2011; Jeong et al., 2013; Scott et al., 2004). The CBSX subfamily of the CBS-containing proteins contains a single CBS pair and likely regulates the function of their interacting targets (Kushwaha et al., 2009). The Arabidopsis genome encodes six CBSX proteins of which CBSX1 and CBSX2 are localized in chloroplasts (Ok et al., 2012). CBSX1 and CBSX2 interact with several Trxs, and both of them were shown to enhance the in vitro activity of chloroplast Trxs including isoforms of *m2*, *f1*, *x*, and *y1* (Yoo et al., 2011). Recently, however, Baudry et al. found that CBSX2 selectively inhibits the activities of *m*-type TRXs, and ATP can reverse this effect (Baudry et al., 2022). In addition, Murai et al. proposed that CBSX1 and CBSX2 may not function as Trx regulator for activation of the Calvin-Benson cycle enzymes FBPase and SBPase in light conditions (Murai et al., 2021). Thus, it is still unclear how the chloroplast CBSX1 and CBSX2 modulate the activity of the Trx systems and their different targets.

In this study, we found that the *CBSX2*-knockout Arabidopsis mutant has altered non-photochemical quenching (NPQ) induction during a dark–light transition. Further analysis showed that oxidation of the $CF_1\gamma$ subunit, Calvin–Benson cycle enzymes FBPase and SBPase are less efficient under these conditions in the mutant. According to the interactome of CBSX2, we propose that CBSX2 is

involved in the oxidation of chloroplast redox-regulated enzymes, probably through the regulation of Trxs, NTRC, and 2-Cys Prx in the dark.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The mutant *cbsx2* was isolated from the pSKI015 T-DNA insertion Arabidopsis mutant pool (stock no.: CS31400). T-DNA insertion mutant *cbsx1* (SAIL_1271_E12) was obtained from NASC (Nottingham Arabidopsis Stock Centre). Plants were grown on soil at 23°C under long-day conditions (16/8-h light/dark at 50 µmol photons m^{-2} s^{-1}). To complement the *cbsx2* mutant, the cDNA of *CBSX2* was fused with the sequence encoding the HA tag (influenza hemagglutinin protein epitope) and then cloned into the pBI121 vector. The binary plasmid (*pBI121–35 s:CBSX2:HA*) was used to transform *cbsx2* mutant by floral dipping.

2.2 | Chlorophyll fluorescence measurements

NPQ induction and PSII quantum yield Y (II) were measured with a default program with a FluorCAM 800-O (Photon System Instruments, Zhang et al., 2016). Plants were dark-adapted for at least 30 min. The maximum chlorophyll fluorescence in the dark was obtained by a saturating light pulse. During illumination with actinic light (AL, 50 µmol photons m^{-2} s^{-1}), saturating light pulses were applied at 9, 36, 64, and 91 s. NPQ and Y (II) images were recorded and extracted using the system software. At least three leaves were selected to derive NPQ and Y (II) data for dynamic analysis upon illumination.

2.3 | Protein extraction and protein gel blot analyses

Thylakoid membrane isolation and 2D-PAGE analysis were performed as previously described (Zhang et al., 2018). Antibodies against D1 (PHY0103, PhytoAB), PsaA (PHY0342, PhytoAB), $CF_1\alpha$ (PHY0312, PhytoAB), and $CF_1\gamma$ (PHY0313, PhytoAB) were obtained from PhytoAB (USA).

2.4 | AMS labeling of CF1γ, SBPase, and FBPase

The redox state of the γ subunit was determined using the specific thiol-labeling reagent 4-acetoamido-4′-maleimidylstilbene-2, 2′-disulfonate (AMS), which only labels the free thiol groups of cysteine residues. The procedure was performed as previously described (Carrillo et al., 2016). Leaves with similar size were ground into powder in liquid nitrogen and then transferred into a 1.5 ml centrifuge tube containing 500 µL of ice-cold 10% (v/v) trichloroacetic acid. The sediment was collected by centrifugation and washed twice with ice-cold acetone. After drying, 40 µL of AMS labeling solution (8 mM AMS, 50 mM Tris–HCl, pH 8.0, and 1% w/v SDS) was added and incubated for 2 h at room temperature in the dark. Proteins in the solution were separated by nonreducing 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and incubated with specific antibodies against the $CF_1\gamma$ subunit, SBPase (PHY0410S, PhytoAB), and FBPase (PHY3095A, PhytoAB). The immunoblot signal was captured by the chemiluminescence imaging system (LuminoGraph WSE-6100; ATTO Technology).

2.5 | Measurement of ΔA515 relaxation kinetics

Four-week-old plants were dark-adapted overnight. The ΔA515–560 nm signal was detected during excitation of the adaxial leaf side by 200,000 µmol photons m^{-2} s^{-1} with a single turnover flash of 5 µs duration using the Dual-PAM-100 system equipped with the P515/535 module (Walz, Germany).

2.6 | Affinity chromatography and mass spectrometry analysis

Freshly isolated intact chloroplasts from WT and *cbsx2-HA*-com plants were cross-linked with 2.5 mM DSP (dithiobis [succinimidyl propionate]) as previous described (Zhang et al., 2016). After crosslinking, Tris–HCl pH 7.5 was added to a final concentration of 60 mM to stop the reaction and chloroplasts were osmotically ruptured in a buffer containing 20 mM HEPES-KOH pH 7.6 and 10 mM $MgCl_2$ supplemented with protein inhibitor cocktail (Roche). Thylakoids were removed by centrifugation twice at 18,000 g for 10 min at 4°C. The supernatant containing chloroplast stromal proteins was incubated with anti-HA MicroBeads (Miltenyi Biotec) for 2 h at 4°C. The bound proteins were purified according to the manufacturer's instructions and separated by SDS-PAGE. The gel strip was then directly used for liquid chromatography-mass spectrometry (LC–MS) analysis.

2.7 | Yeast two-hybrid (Y2H)

Y2H analysis was performed following the MatchmakerTM Gold Y2H System procedure (Clontech, Mountain View, CA, USA). The sequences encoding the mature NTRC and CBSX2 proteins were fused into the GAL4 activation domain vector (pGADT7) and the GAL4 binding domain vector (pGBKT7), respectively. The resulting vectors were cotransformed into Y2HGold yeast strain by the small-scale lithium acetate method. Positive colonies grown on double-dropout medium (SD/-Leu/-Trp) were cultured on tripartite-dropout (SD/-Leu/-Trp/-His) and quadruple-dropout (SD/-Leu/-Trp/-His/-ade) medium in the presence of X-α-Gal to investigate protein–protein interactions.

3 | RESULTS

3.1 | Isolation and identification of the *cbsx2* mutant

The mutant *cbsx2* was identified within a pSKI015 T-DNA insertion mutant pool of Arabidopsis (Figure 1a). This mutant shows different NPQ induction after illumination compared with wild-type (WT) plants (Figure 1b,c). During transition from dark to light, activation of photosynthetic electron transport induces accumulation of protons in the thylakoid lumen. Acidification of the thylakoid lumen subsequently triggers the induction of the main component of NPQ, qE, which provides efficient photo-protection (Niyogi, 1999). ATP synthase is inactivated in darkness and its full activation upon illumination occurs after a lag period. Thus, during transition from dark to light, NPQ was rapidly and transiently induced to 0.8 after illumination for 40 s in WT (Figure 1b,c). Proton efflux from the thylakoid lumen through ATP synthase occurs along with the activation of ATP synthase upon illumination. Thus NPQ was rapidly relaxed within 2 min of illumination of the WT (Figure 1b,c). In the *cbsx2* mutant, the induction of NPQ was higher than in WT after illumination for 9 s. However, the NPQ level in *cbsx2* was lower than in WT after illumination for approximately 36 and 64 s, indicating that relaxation of NPQ is more efficient in the *cbsx2* mutant (Figure 1b,c). The *cbsx2* mutant did not exhibit a visible growth phenotype under growth chamber conditions, and the maximum photochemical efficiency (Fv/Fm) was comparable to that of WT (Figure 1b) indicating that photosystem II (PSII) function in *cbsx2* was not affected. However, we also found that the quantum yield of photosystem II [Y (II)] of *cbsx2* was higher than in WT after illumination for 40 s (Figure 1b,d), suggesting that the photosynthetic electron transfer of *cbsx2* was enhanced upon illumination.

Map-based cloning and resequencing of the *cbsx2* genome DNA revealed that 14 nucleotides are missing in the *CBSX2* (*AT4G34120*) gene (Figure 1a). To confirm that the mutation of *CBSX2* is responsible for the NPQ induction phenotype, we introduced the WT *AT4G34120* cDNA sequence into *cbsx2* (*cbsx2* com), which fully complemented the *cbsx2* phenotype (Figure 1). These results indicate that the loss of the 14 nucleotides of *CBSX2* is responsible for the NPQ phenotype in the *cbsx2* mutant.

3.2 | Arabidopsis *cbsx1* mutant does not have the same NPQ phenotype as *cbsx2*

A total of six CBSX proteins were found to have only one pair of CBS domains in Arabidopsis, and only CBSX1 and CBSX2 are localized in chloroplasts (Ok et al., 2012). To test whether *cbsx1* exhibits the same NPQ phenotype as *cbsx2*, we analyzed the NPQ induction upon illumination of the single *cbsx1* mutant and the *cbsx1cbsx2* double mutant. As shown in Figure 2, both the single *cbsx1* mutant and the double *cbsx1cbsx2* mutant did not exhibit a visible growth phenotype under growth chamber conditions (Figure 2a). The induction of NPQ in *cbsx1* is similar to that of WT (Figure 2). Moreover, the NPQ induction curve in the double *cbsx1cbsx2* mutant shows identical defects as the single *cbsx2* mutant (Figure 2). These results indicate that in contrast to CBSX2, CBSX1 is not involved in the induction and relaxation of NPQ.

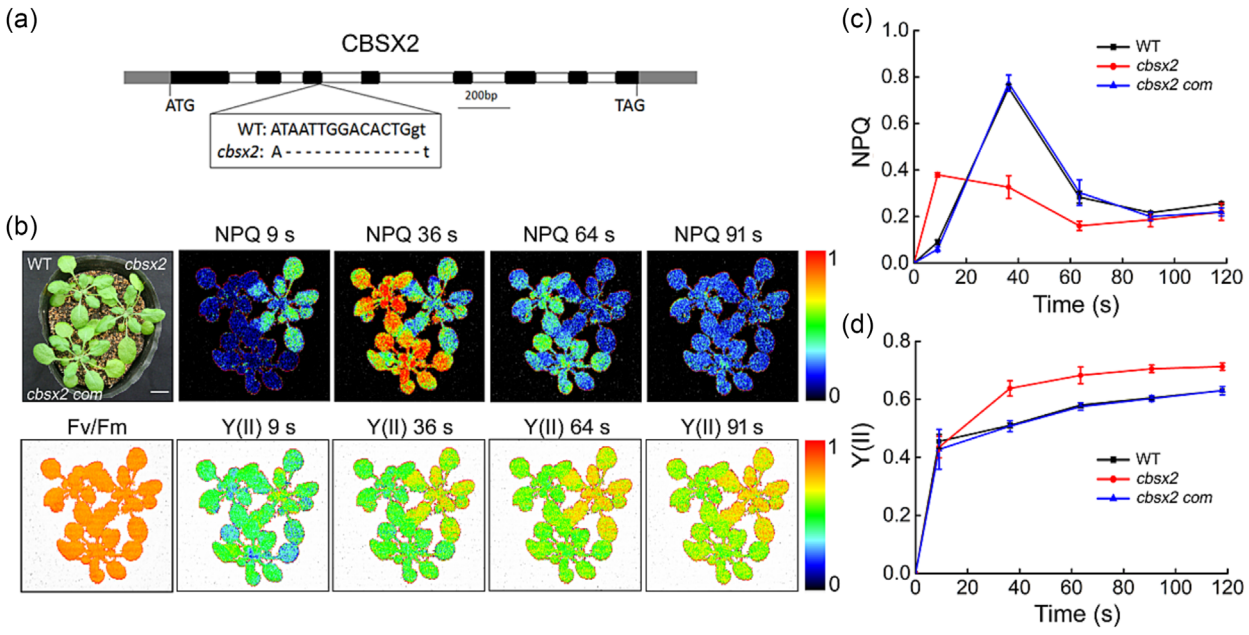

FIGURE 1 Phenotype of the *cbsx2* mutant. (a) Schematic representation of *CBSX2*. The *cbsx2* mutant has a deletion of 14 bases in *CBSX2* (*AT4G34120*). (b) Growth, non-photochemical quenching (NPQ), maximum photochemical efficiency (Fv/Fm), and PSII quantum yield Y (II) phenotypes of WT, *cbsx2*, and *cbsx2 com*. Four-week-old plants grown on soil at 50 µmol photons m^{-2} s^{-1} (left panels). Signal intensities for NPQ and Y (II) at various times upon illumination with AL 50 µmol photons m^{-2} s^{-1} and Fv/Fm are indicated according to the color scale (0 to 1.0) on the right. Time course of NPQ induction (c) and Y (II) (d) of WT, *cbsx2*, and *cbsx2 com*. Data are presented as the means ± SD ($n = 3$).

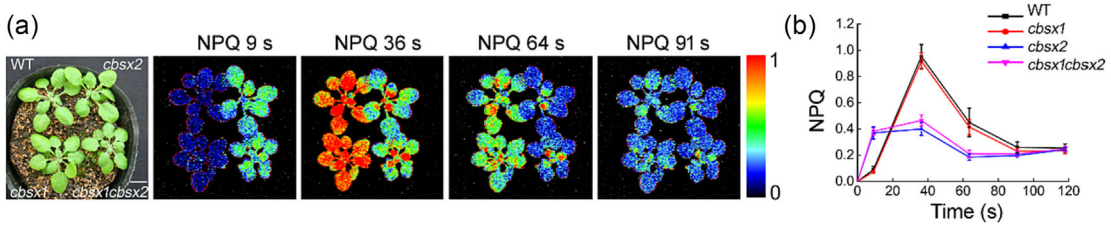

FIGURE 2 Growth and non-photochemical quenching (NPQ) phenotypes of WT and *cbsx* mutants. (A) Growth and NPQ phenotypes of WT, *cbsx2*, *cbsx1* and *cbsx1cbsx2*. (B) NPQ induction curves of WT, *cbsx2*, *cbsx1*, and *cbsx1cbsx2*. The AL intensity is 50 μmol photons m^{-2} s^{-1}, data are presented as the means ± SD ($n = 4$).

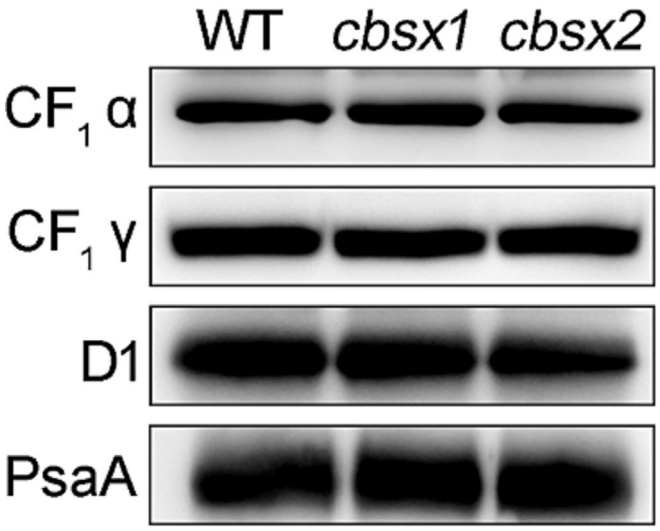

FIGURE 3 Thylakoid protein gel blot analysis in *cbsx1* and *cbsx2*. Freshly isolated thylakoid membrane proteins were separated by SDS-urea-PAGE and immunodetected with the antibodies against $CF_1\alpha$, $CF_1\gamma$, D1, and PsaA.

3.3 | Accumulation of the chloroplast ATP synthase is not affected in the *cbsx2* mutant

The above NPQ induction properties suggest that chloroplast ATP synthase activity or accumulation is affected in the *cbsx2* mutant. To distinguish between these two possibilities, we first analyzed the accumulation of the ATP synthase in *cbsx2*. Immunoblot analysis showed that the levels of the chloroplast ATP synthase subunits $CF_1\alpha$ and $CF_1\gamma$ are similar in *cbsx1*, *cbsx2*, and WT plants (Figure 3), indicating that accumulation of the chloroplast ATP synthase is not affected in the absence of the CBSX1 and CBSX2 proteins.

3.4 | Oxidation of the $CF_1\gamma$ subunit in darkness is less efficient in the *cbsx2* mutant

Since the ATP synthase level was not altered in the *cbsx2* mutant, CBSX2 may be involved in the reduction of the γ subunit of the chloroplast ATP synthase. To investigate the redox status of $CF_1\gamma$ in the WT and *cbsx2* plants, we first analyzed the redox state of $CF_1\gamma$ in the dark and light. Reduced and oxidized $CF_1\gamma$ subunit could be separated and quantified by the AMS labeling method (Konno et al., 2012). Immunoblot analyses showed that $CF_1\gamma$ subunit from WT plants was completely oxidized in the dark, but a small amount of $CF_1\gamma$ subunit in *cbsx2* remained in the reduced state (Figure 4a). Under light conditions, most of the $CF_1\gamma$ subunits were present in the reduced form in WT and *cbsx2*, and identical levels of the redox forms of the $CF_1\gamma$ subunit were detected in these two genotypes (Figure 4a).

The redox regulation of $CF_1\gamma$ subunit in plants mainly occurs in the reciprocal transition between darkness and light, so we examined the redox changes of $CF_1\gamma$ subunit in WT and *cbsx2* leaves during the transition between darkness and light. As shown in Figure 4b, $CF_1\gamma$ subunit of WT is completely oxidized within 4 min after transition from light to darkness. However, a substantial amount of reduced $CF_1\gamma$ is still present in *cbsx2* even for 6 min after transition from light to darkness (Figure 4b). The $CF_1\gamma$ subunit can be rapidly reduced within a short period of time upon illumination to produce the active ATP synthase in light. No significant difference was detected in the reduction rate of $CF_1\gamma$ subunit between WT and *cbsx2* (Figure 4c). These results indicate that the oxidation of $CF_1\gamma$ subunit in darkness is less efficient and the reduction of $CF_1\gamma$ in light is not affected in the absence of CBSX2.

Plants must turn off the chloroplast ATP synthase at night to prevent the hydrolysis of ATP when the chloroplast redox potential cannot sustain ATP synthesis (Kühlbrandt, 2019). However, a substantial amount of reduced $CF_1\gamma$ is present in the *cbsx2* leaves even after staying overnight in darkness (Figure 4a), suggesting that a portion of chloroplast ATP synthase is still active in darkness. To test this possibility, we measured the changes in the electrochromic pigment absorbance shift (ECS) signal (ΔA515) caused by a single flip saturation flash using the leaves dark-adapted for at least 12 h. As shown in Figure 4d, the ΔA515 signal did not significantly relax within 80 ms in WT leaves, indicating that the ATP synthase is inactive after dark adaption overnight. In contrast, the ΔA515 signal relaxed slowly in the *cbsx2* leaves (Figure 4d), suggesting that the protons generated by the saturation flash flow out the thylakoid lumen slowly through the active ATP synthase. This conclusion is consistent with the presence of the reduced form of $CF_1\gamma$ in the *cbsx2* leaves dark-adapted overnight (Figure 4a).

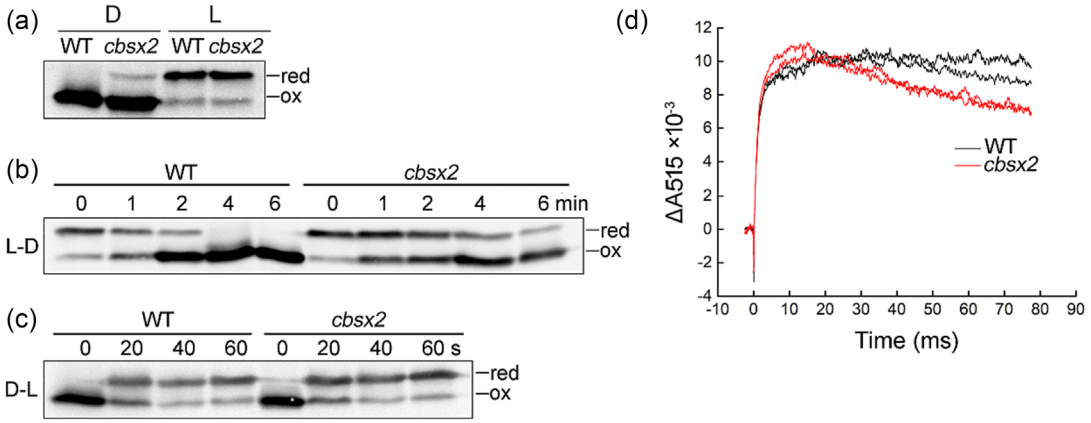

FIGURE 4 The redox state of $CF_1\gamma$ in WT and *cbsx2* leaves. (a) The redox status of $CF_1\gamma$ in leaves under light (L, 50 µmol photons m^{-2} s^{-1}) and dark conditions (D). Proteins were extracted from leaves dark-adapted overnight at least for 8 h (D) and illuminated (L). The proteins were treated with AMS and separated by SDS-PAGE for immunoblot analyses with an antibody against $CF_1\gamma$. (b) Changes in redox status of $CF_1\gamma$ in leaves transferred from light to darkness (L-D). The leaves subjected to light were dark-adapted for various times indicated above and proteins were analyzed as in (a). (c) Changes in redox status of $CF_1\gamma$ in leaves transferred from darkness to light (D-L). Overnight dark-adapted leaves were illuminated for various times as indicated above and proteins were analyzed as in (a). (d) ΔA515 relaxation kinetics in WT and *cbsx2* leaves. The changes in the ECS signal (ΔA515) was measured using Dual-PAM-100 with a P515/535 module. The signal was detected during excitation of the adaxial leaf side by 200,000 µmol photons m^{-2} s^{-1} with a single turnover flash of 5 µs duration.

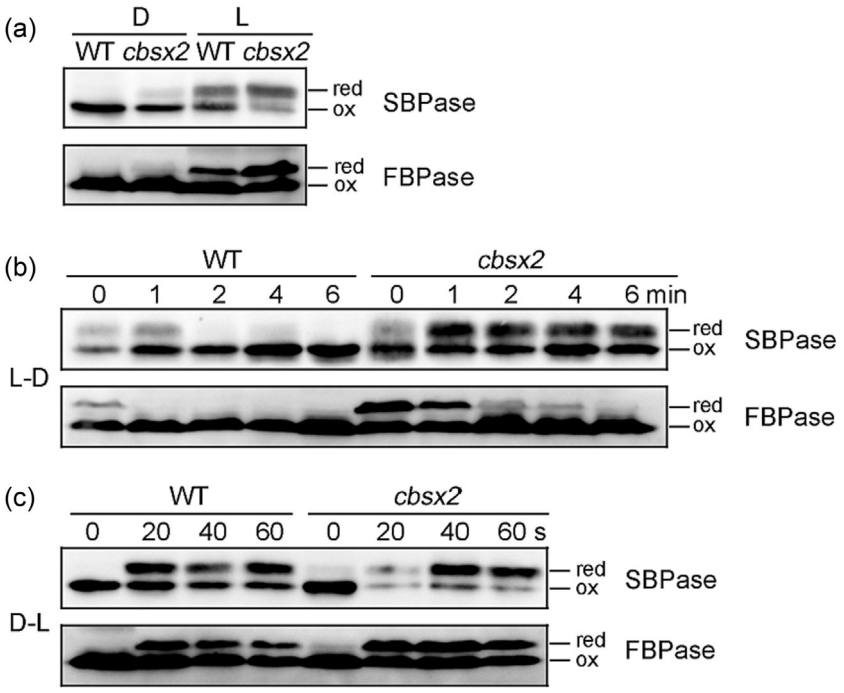

FIGURE 5 The redox state of SBPase and FBPase in WT and *cbsx2* leaves. (a) The redox status of SBPase and FBPase in leaves under light (L, 50 µmol photons m^{-2} s^{-1}) and dark conditions (D). Proteins were extracted from leaves dark-adapted overnight at least for 8 h (D) and illuminated (L). The proteins were treated with AMS and separated by SDS-PAGE for immunoblot analyses with antibodies against SBPase and FBPase. (b) Changes in redox status of SBPase and FBPase in leaves transferred from L-D. (d) Changes in redox status of SBPase and FBPase in leaves transferred from D-L.

3.5 | Oxidation of the Calvin cycle enzymes SBPase and FBPase in darkness is also less efficient in the *cbsx2* mutant

We also examined redox changes of the Calvin cycle enzymes SBPase and FBPase in WT and *cbsx2* leaves. Immunoblot analysis showed that FBPase and SBPase are completely oxidized in WT plants in the dark, but a small amount of FBPase and SBPase are reduced in *cbsx2* (Figure 5a). After transition from light to darkness, SBPase and FBPase in WT plants are completely oxidized within 2 and 1 min, respectively (Figure 5b). In contrast, a large amount of reduced SBPase and a trace amount of FBPase are still present in *cbsx2* even 6 min after transition from light to darkness (Figure 5b). During transition from dark to light, a part of FBPase and SBPase are rapidly reduced both in WT and *cbsx2* plants (Figure 5c). These results indicate that CBSX2 is also involved in the oxidation of the Calvin cycle enzymes FBPase and SBPase in darkness.

3.6 | CBSX2 coimmunoprecipitates with components of several chloroplast redox systems

CBSXs have been identified as redox regulators of the thioredoxin system through their interaction with several Trxs (Yoo et al., 2011). To investigate the potential interaction partners of CBSX2, coimmunoprecipitation (Co-IP) experiments were performed. We complemented the *cbsx2* mutant with a chimeric gene encoding the CBSX2 protein carrying a HA (influenza hemagglutinin protein epitope) tag. The CBSX2-HA protein and its interacting proteins were affinity purified from CBSX2-HA plants using the mMACS HA isolation kit (Miltenyi Biotec). Total purified protein was separated by gel electrophoresis and further analyzed by LC–MS/MS analysis. The results showed that in addition to the thioredoxin (Trxs) members (*f1*, *m1*, *m4*, *f1*, *y2*, and CDSP32/Trx L1), peroxiredoxins (2CPA, 2CPB, PrxIIE, and PrxQ) were identified in the CBSX2-HA-IP sample (Table 1). Besides these proteins, NADPH-dependent thioredoxin reductase C (NTRC), ferredoxin-dependent glutamate synthase (GLU1, GLU2), SIR (sulfite reductase), ferredoxin-nitrite reductase (NIR1), and ferredoxin-NADP reductase (RFNR1, RFNR2, LFNR1, and LFNR2) were also co-purified (Table 1). These results suggest that CBSX2 interacts with a wide range of substrates in vivo and participates in the regulation of multiple chloroplast redox systems.

3.7 | CBSX2 interacts with NTRC

A previous report showed that NTRC regulates the activity of the chloroplast ATP synthase through reduction of the γ subunit (Nikkanen et al., 2016). NTRC is a NADPH-dependent Trx reductase with a joint Trx domain and was co-purified with CBSX2 (Table 1). Nikkanen et al. also detected an interaction between $CF_1\gamma$ and NTRC by Co-IP experiments (Nikkanen et al., 2016). These results suggest that CBSX2 may regulate chloroplast ATP synthase activity via NTRC. To investigate whether CBSX2 directly interacts with NTRC, we performed Y2H experiments. As expected, yeast expressing the NTRC and CBSX2 can growth on the tripartite-dropout and quadruple-dropout mediums (Figure 6), indicating a physical interaction between CBSX2 and NTRC in yeast.

4 | DISCUSSION

The activity of CF_oCF_1-ATP synthase is reversible as it catalyzes both the synthesis and hydrolysis of ATP. In the absence of the light reactions that produce *pmf* across the thylakoid membrane, the enzyme is inactivated, preventing futile ATP hydrolysis in the dark. When the $CF_1\gamma$ thiol is oxidized, a rather high threshold *pmf* is required to activate the enzyme, and the break of the disulfide bridge in the $CF_1\gamma$ subunit reduces the threshold *pmf* required for activation (Hisabori et al., 2013; Konno et al., 2012; Nikkanen et al., 2016). The *pmf* across the thylakoid membrane includes the proton gradient (ΔpH) and the electric membrane potential ($\Delta\Psi$). The ΔpH is a key signal for triggering the induction of NPQ of PSII, which is crucial for the dissipation of excess absorbed light energy to protect against photochemical damage of PSII (Wang et al., 2022). The altered NPQ induction pattern upon illumination in *cbsx2* can be explained by the redox state of $CF_1\gamma$ in this mutant (Figures 1 and 4). In darkness, a small amount of

TABLE 1 Redox related proteins detected in the CBSX2-HA-IP sample by IP-MS.

Protein name		Gene no.	Coverage	Unique peptides
Trx *f1*	Thioredoxin f1	At3g02730	6.74	1
Trx *m1*	Thioredoxin m1	At1g03680	13.97	2
Trx *m2*	Thioredoxin m2	AT4G03520	56.94	3
Trx *m4*	Thioredoxin m4	At3g15360	16.58	3
Trx *y2*	Thioredoxin y2	AT1G43560	4.79	1
CDSP32/Trx L1	Thioredoxin like protein	AT1G76080	11.26	3
2-Cys Prx A/2CPA	2-Cys peroxiredoxin A	AT3G11630	52.26	5
2-Cys Prx B/2CPB	2-Cys peroxiredoxin B	At5g06290	32.6	1
PrxIIE	Peroxiredoxin-II-E	AT3G52960	29.06	6
PrxQ	Peroxiredoxin Q	AT3G26060	11.11	2
NTRC	NADPH-dependent thioredoxin reductase C	AT2G41680	44.61	23
GLU1	Ferredoxin-dependent glutamate synthase 1	AT5G04140	34.28	35
GLU2	Ferredoxin-dependent glutamate synthase 2	AT2G41220	29.1	29
SIR	Sulfite reductase	AT5G04590	36.92	23
NIR1	Ferredoxin-nitrite reductase	AT2G15620	37.71	22
RFNR1	Root-type ferredoxin: NADP(H) oxidoreductase 1	AT4G05390	19.84	4
RFNR2	Root-type ferredoxin: NADP(H) oxidoreductase 2	AT1G30510	8.12	2
LFNR1	Leaf-type ferredoxin: NADP(H) oxidoreductase 1	AT5G66190	10	3
LFNR2	Leaf-type ferredoxin: NADP(H) oxidoreductase 2	AT1G20020	3.25	1

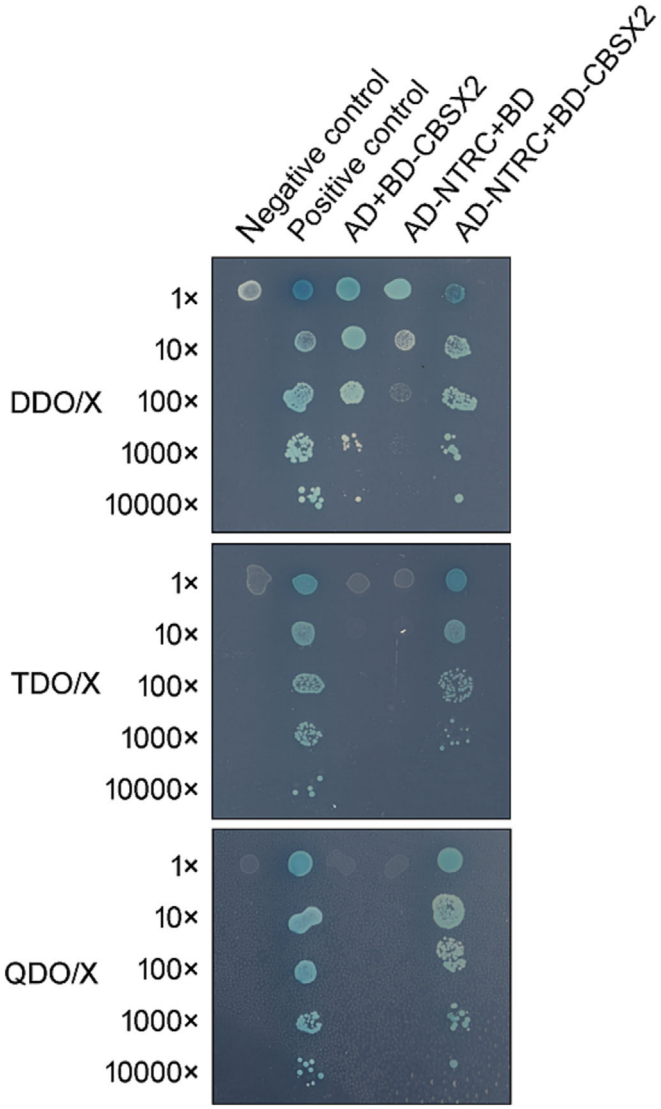

FIGURE 6 Yeast two-hybrid assay for the interaction between CBSX2 and NTRC. AD-NTRC, the mature NTRC protein fused to the GAL4 activation domain (AD). BD-CBSX2, the mature CBSX2 protein fused into the GAL4 binding domain (BD). Cotransformation of pGBKT7-53 with pGADT7-T and pGBKT7-Lam with pGADT7-T were used as positive and negative controls, respectively. Cotransformation of AD with BD-CBSX2 and BD with AD-NTRC were used to verify no self-activation of the prey or bait. DDO/X, TDO/X, and QDO/X represent double-dropout, tripartite-dropout, and quadruple-dropout medium containing X-α-Gal, respectively. Numbers represent the dilution of the yeast culture from 1× to 10,000×.

reduced $CF_1\gamma$ was detected in the *cbsx2* mutants (Figure 4a). Thus, it is likely that ATP hydrolysis occurs, which in turn leads to a flow of protons from chloroplast stroma to thylakoid lumen, resulting in a higher level of ΔpH across the thylakoid membrane in *cbsx2* than in WT in the dark. Consequently, a higher NPQ was induced in *cbsx2* compared with WT in the first few seconds after the transition from dark to light (Figure 1b,c). However, upon continued illumination, proton efflux from the thylakoid lumen through ATP synthase was higher in *cbsx2* compared with WT, probably due to the higher initial CF_oCF_1-ATP synthase activity in *cbsx2* than in WT and because full activation of CF_oCF_1-ATP synthase upon illumination needs some time in WT. Therefore, NPQ is lower in *cbsx2* than WT after light for about 35 s (Figure 1b,c). Similar levels of NPQ were detected for WT and *cbsx2* upon illumination for 120 s (Figure 1b,c), which is consistent with the previous finding that there is no significant change of ETR and NPQ in *cbsx2* during steady-state photosynthesis under different light intensities (Murai et al., 2021).

We also found that Y (II) is higher in *cbsx2* than WT after the transition from dark to light for about 40 s (Figure 1b,d), indicating enhanced electron transport through PSII in *cbsx2*. Analyses of the redox state of Calvin-Benson cycle enzymes SBPase and FBPase in WT and *cbsx2* leaves showed that a small amount of reduced SBPase and FBPase was detected in the *cbsx2* mutants in darkness (Figure 5a). These results suggest that the Calvin-Benson cycle enzymes are not fully inactivated in the dark in the absence of CBSX2. Reducing power generated by photosynthetic electron transport may be more efficiently transferred to the Calvin-Benson cycle in *cbsx2* than in WT plants, thus enhancing the quantum yield of photosystem II. It is also possible that enhanced Y (II) resulted from the reduced NPQ level during the transition from dark to light (Figure 1b,c).

Oxidation of $CF_1\gamma$, FBPase, and SBPase is less efficient during a transition from light to darkness in the *cbsx2* mutant (Figures 4 and 5), suggesting that CBSX2 generally participates in the oxidation of chloroplast redox-regulated proteins including Calvin cycle enzymes and $CF_1\gamma$ in the dark and during a transition from light to dark. Our IP-MS results showing that several components of the chloroplast redox systems including peroxiredoxin (2CPA, 2CPB, PrxIIE, and PRXQ), Trx *f*, Trx *m*, CDSP32/Trx L1 are coimmunoprecipitated with CBSX2 (Table 1). Among them, Trx *f* and Trx *m* are thought to be modulators of thiol enzymes in the Calvin-Benson cycle (Michelet et al., 2013; Murai et al., 2021). An impaired reoxidation of redox-regulated proteins during the light–dark transition was found in the Arabidopsis plants with decreased 2-Cys Prx (2CP) levels (Yoshida et al., 2018). Moreover, about half of $CF_1\gamma$ remained in the reduced form in the *2cpa 2cpb* double mutants (Yoshida et al., 2018). PRXQ and PRXIIE may act as Trx oxidases in regulating photosynthesis and chloroplast metabolism similar to 2CP (Telman et al., 2020). CDSP32/Trx L1 mediates electron transfer from Fd to 2CP in chloroplasts, playing a role in assisting 2CP to clear H_2O_2 (Broin & Rey, 2003). Recently, it has been shown that Trx *f* and two types of Trx-like proteins, Trx-L2 and ACHT (atypical Cys-His-rich Trx), are involved in the oxidation of different Trx-targeted proteins, such as glyco-1, 6-diphosphatase, Rubisco activase, and the γ subunit of ATP synthase (Sekiguchi et al., 2022; Yokochi et al., 2021). In contrast to the FTR-Trx system, NTRC appears to function primarily in the dark or under limited light, possibly because it derives its reducing potential from NADPH, which can be produced independently of photosynthesis through the pentose phosphate oxidation pathway (Carrillo et al., 2016). The NTRC activity was proposed to be modulated between light and dark conditions (Carrillo et al., 2016). Nikkanen et al. (2016) confirmed the interaction between $CF_1\gamma$ and NTRC by CoIP, and CBSX2 was shown to

interact with NTRC by bimolecular fluorescence complementation assays (González et al., 2019). We also confirmed the direct interaction between CBSX2 and NTRC by IP-MS and Y2H assays (Figure 6; Table 1). Similar with the *cbsx2* mutant, a small amount of $CF_1\gamma$ is also present in the reduced form in plants with overexpressed NTRC (Nikkanen et al., 2016; Figure 4a). Recently, in vitro biochemical studies confirmed that CBSX protein acts as a negative regulator of NTRC in the presence of AMP, and even low concentration of CBSX protein can significantly inhibit the activity of NTRC (Tran et al., 2023). Thus, we propose that CBSX2 is involved in the oxidation of a large set of chloroplast redox-regulated enzymes by modulating the activity of these chloroplast redox systems in vivo.

AUTHOR CONTRIBUTIONS

Lianwei Peng and Fudan Gao conceived the study and designed the experiments. Yonghong Li, Yurou Shen, and Lin Zhang performed the experiments and produced the figures. Yonghong Li, Lianwei Peng, and Fudan Gao wrote the manuscript. All authors analyzed the data. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank the NASC and ABRC for providing the mutant seeds.

CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Li, Y., Zhang, L., Shen, Y., Peng, L., & Gao, F. (2023). CBSX2 is required for the efficient oxidation of chloroplast redox-regulated enzymes in darkness. *Plant Direct*, *7*(11), e542. https://doi.org/10.1002/pld3.542